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14. ABSTRACT Obesity has become a major health problem in children and adults and is associated with increased breast cancer incidence and mortality. The epidemic of childhood obesity is recent and little information exists regarding its association with mammary tumorigenesis. Towards better understanding this relationship we have developed and characterized a new rat model of childhood onset Diet Induced Obesity (DIO) and breast cancer. We have shown that young female rats fed a high fat Western Style diet have a 2-fold higher body fat mass and elevated serum comorbidity factors as compared to Chow fed Lean rats. When these animals are treated with the carcinogen MNU, mammary tumors appear sooner and in greater numbers in Obese rats. We determined via histology that tumors from Obese rats are of a more invasive type compared to tumors from Lean rats. This is in accord with the association between human obesity and breast cancer mortality. This new model parallels the onset of obesity as it occurs in humans and therefore provides an excellent system to study the underlying mechanisms of obesity and mammary tumor formation and progression. Our long-term goals are to exploit this model to better understand adipocyte-epithelial interactions during mammary tumorigenesis, identify and validate novel molecular therapeutic targets, and to establish biomarkers for cancer prevention and prognosis.

15. SUBJECT TERMS
 Obesity, Breast Cancer, Western Diet, rat, MNU

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Introduction

Obesity is a major health problem around the world and is positively associated with breast cancer incidence and mortality (1). Recent rapid increases in childhood obesity indicate the adverse effects of obesity will be a concern for decades to come (2). To better understand the relationship between childhood obesity and breast cancer, we have developed and characterized a new rat model of early onset Diet Induced Obesity (DIO). In this model rats are fed a Western Diet that is high in fat and higher in simple carbohydrate compared to diets of traditional DIO studies. This more closely represents the typical human diet of North America. We have shown that young female rats fed a Western diet have a higher body fat mass and elevated serum comorbidity factors as compared to Chow fed Lean rats. Furthermore, MNU-induced mammary tumors appear sooner, in greater numbers and are more invasive in Obese rats as compared to tumors from Lean rats. This is in accord with the association between human obesity and breast cancer mortality. This new model provides an excellent system to identify the mechanisms of obesity towards mammary tumorigenesis, to better understand adipocyte-epithelial interactions and to establish biomarkers for cancer prevention and prognosis.

Body

Development of a Novel Rat model of Diet Induced Obesity (DIO) – S.O.W. Task 1

Rational for a New model of Female Obesity

In humans obesity is associated with increased breast cancer incidence and morbidity (1). To better understand the mechanisms involved in obesity-associated breast cancer, it is essential to establish a model of obesity that parallels the human condition. In our recently developed model, out bred female Sprague-Dawley rats are fed a "Western Diet" beginning at weaning. This model was chosen for several reasons. First, the Western diet used is high in fat and higher in simple carbohydrates as compared to traditional high fat diets used in animal studies and more closely represents a typical human diet of North America and Europe (Table 1). Second, the most widely used rodent models of obesity are genetically altered and devoid of the obesity hormone leptin (*ob/ob* mice) or its receptor (*db/db* mice, Zucker rat). These leptin signaling impaired animals are resistant to oncogene and chemically induced mammary tumors (3,4). However, human obesity is not generally caused by mutations in leptin or its receptor (5). As expression of leptin and its receptor remain intact in our rat model, it more closely represents human obesity. Third, in this model obesity is determined by Dual Xray Absorbimetry (DEXA) scan and not by body weight. This method allows the measurement of body composition of animals, in particular % body fat mass. Given that two animals having the same weight can differ vastly in their adiposity, DEXA scan provides a substantial advantage compared to scale weight measurements. Finally, the model we have developed mimics childhood obesity in humans, a trend that has dramatically increased in recent years (2). Compared to lean children, children who are obese are far more likely to become obese adults. This disturbing trend is an indicator that obesity-related cancers will be a concern for decades to come.

Proportion of rats susceptible to DIO

Current data, based on BMI, reveals that 30% of the American population is considered clinically obese (6). Given their similarity in physiology and genetics to humans, it is likely that within a population of outbred rats, a proportion of rats would be susceptible to DIO. Indeed this was shown to be true by Ghibaudi *et al.* (7) where outbred Sprague-Dawley male rats were fed a Western diet beginning at 50 days old. After 6 weeks, approximately 15-30% of the Western diet fed rats gained a significantly higher amount of weight compared to control fed animals. Based on this study, we tested if this phenomenon could occur in female Sprague-Dawley rats. Establishing that the Western diet can consistently induce obesity in a subpopulation of outbred female Sprague-Dawley rats was essential towards establishing our *in vivo* model of obesity-associated breast cancer. In our preliminary study, female Sprague-Dawley rats weaned at 21 days old were randomly placed on either a standard Rat Chow Diet (LabDiet) or a Western Diet (Diet Research), shown in Table 1.

	Western	Medium Fat	High Fat
Protein	**17**	**17**	**20**
Casein	16.6	16.5	19.7
Carbohydrate	**43**	**51**	**35**
Corn starch	4.3	18.7	7.2
Sucrose	29.1	25.2	17
Maltodextrin	8.5	6.5	10
Fat	**41**	**32**	**45**
Milk fat	38.4	0	0
Corn oil	2	23.1	0
Soybean oil	0	0	5.5
Lard	0	0	39.5
Butter fat	0	8.7	0

Table 1. Major composition of commonly used DIO Diets by calories in percent. Comparison of Western Diet with representative medium and high fat diets (Ghibaudi, et al., Obesity Research, pp 956-963 10(9):2002). Total percent kilocalories as protein, carbohydrate and fat are broken down by dietary composition. Kcal/g Western Diet = 4.7, Medium Fat Diet = 4.41, and High Fat Diet = 4.73.

All rats were weighed 2 times a week and their food intake was monitored until 54 days of age. Animals were then DEXA scanned (Dual Xray absorbimetry) to determine body fat mass composition. As shown in Table 2A, rats were ranked and then grouped into three categories based on % body fat mass, Obese W = Obese rats on western diet; Lean W = Lean rats on western diet; Lean RC = Lean rats on rat chow diet which served as the Control group. In our DEXA scan data (Table 2A) we show that the Western diet used induces a statistically significant increase in body fat mass as compared to the Rat Chow diet. Approximately 30% of the Western Diet fed rats had a 2-fold increase in % body fat mass as compared to Chow fed rats. Interestingly, approximately 30% of the Western fed rats had the same % fat mass (designated Lean Western) as the Rat Chow fed animals. In an identical but separate study we weighed, monitored food intake and DEXA scanned Western and Chow fed rats after 25 weeks on respective diets. Results from this study demonstrate that a 2-fold difference in body fat mass between Obese Western and Lean Rat Chow animals is maintained through 6 months (Table 2B). As seen with the shorter study, a subset of the Western fed animals had a statistically similar % fat mass as the Rat Chow rats. Figure 1 indicates that after 25 weeks on their respective diets, Obese Western rats have a higher body weight as compared to the Lean Rat Chow Group. Figure 2 demonstrates that Rat Chow fed animals consume more food in grams, compared to the Western fed group. However, when food intake is expressed as Kcal, we show that rats in each group consume the same amount of calories per day. This suggests that the obesity induced in Obese rats in not the result of behavioral differences such as hyperphagia. To further characterize our model, we also measured several circulating co-morbidity factors associated with human obesity in the three groups of rats, including Leptin, Free fatty acids (FFA), triglycerides (TG) and insulin. Figure 3 shows that Obese Western rats had 2-3 fold higher serum leptin and higher TG, FFA and insulin levels as compared to Lean RC animals. The information derived from these preliminary studies was essential in planning the subsequent mammary tumorigenesis experiments. We determined the number of Obese rats that could be produced using the Western diet. We also demonstrated that we could see significant differences in % fat mass between groups at 54 days, the optimal age of effectiveness for inducing mammary tumors with MNU (11).
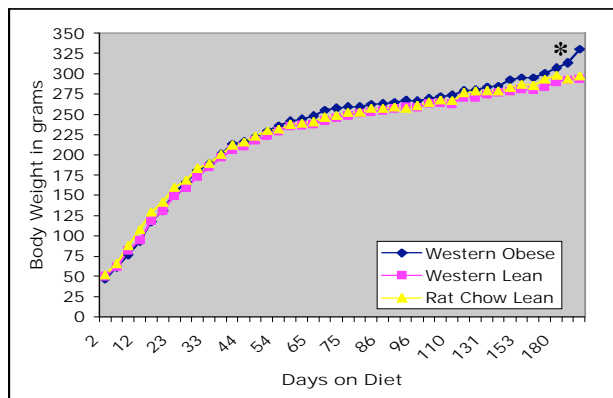
Figure 1. Body weight gain in Western Obese, Western Lean and Rat Chow Lean groups. Body weight was measured bi weekly for 6 1/2 months. At this time rats were DEXA scanned and placed into respective groups based on % body fat mass. Data shown is mean ± sem from day 1 of Western or Rat Chow Diet regimens. n= 12-15 for each group, *p ≤ 0.05.
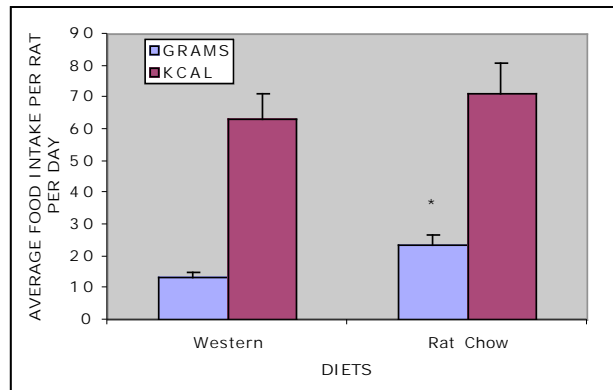
Figure 2. Average Food and energy Intake of female SD rats on Western and Rat Chow diets. Food intake as total grams per rat per day and as Kcal/g metabolizable energy intake per rat per day over 6 1/2 months. Data represents Mean ± sem, in Rats on Western Diet consumed significantly less food intake (in grams) as compared to Rat Chow Diet fed animals, *p ≤ 0.05.

A

Diet	Scale BW(g)	DXA BW(g)	Bone Mass(g)	Fat Mass (%)	Lean Mass (%)
Obese W	185.4 ± 5.6	200.8 ± 5.3	2.5 ± 0.2	**8.6 ± 1.0**	**91.4 ± 1.0**
Lean W	171.7 ± 7.3	179.3 ± 7.2	2.7 ± 0.2	4.2 ± 0.1*	95.8 ± 0.1*
Lean RC	162.8 ± 4.0	176.4 ± 4.3	2.8 ± 0.2	4.0 ± 0.1*	96.0 ± 0.0*

B

Diet	Scale BW(g)	DEXA BW(g)	Bone Mass(g)	Fat Mass (%)	Lean Mass (%)
Obese W	320.3 ± 9.0	338.8 ± 5.9	8.5 ± 0.4	**17.4 ± 1.1***	**82.6 ± 1.1***
Lean W	293.6 ± 4.2	315.2 ± 3.7	8.0 ± 0.3	9.2 ± 1.2	90.8 ± 1.2
Lean RC	297.1 ± 2.9	316.4 ± 4.0	8.5 ± 1.6	9.0 ± 1.6	91.0 ± 1.6

Table 2. Body Mass Composition in Western and Chow Fed Rats. The above table summarizes the body mass composition of our three categories of rats after 54 days **(A)** and 25 weeks **(B)** of diet regimens. Obese W = Obese rats on western diet: Lean W = Lean rats on western diet: Lean RC = Lean rats on rat chow diet; BW = body weight. The first and second columns represent weight of rats as determined by triple beam scale and Dual Xray absorbimetry (DEXA), respectively. Bone mass is represented in grams whereas fat mass and lean mass are expressed as percents. Values are mean ± SEM n = 12-15 per group * p < 0.05 between obese and lean groups.
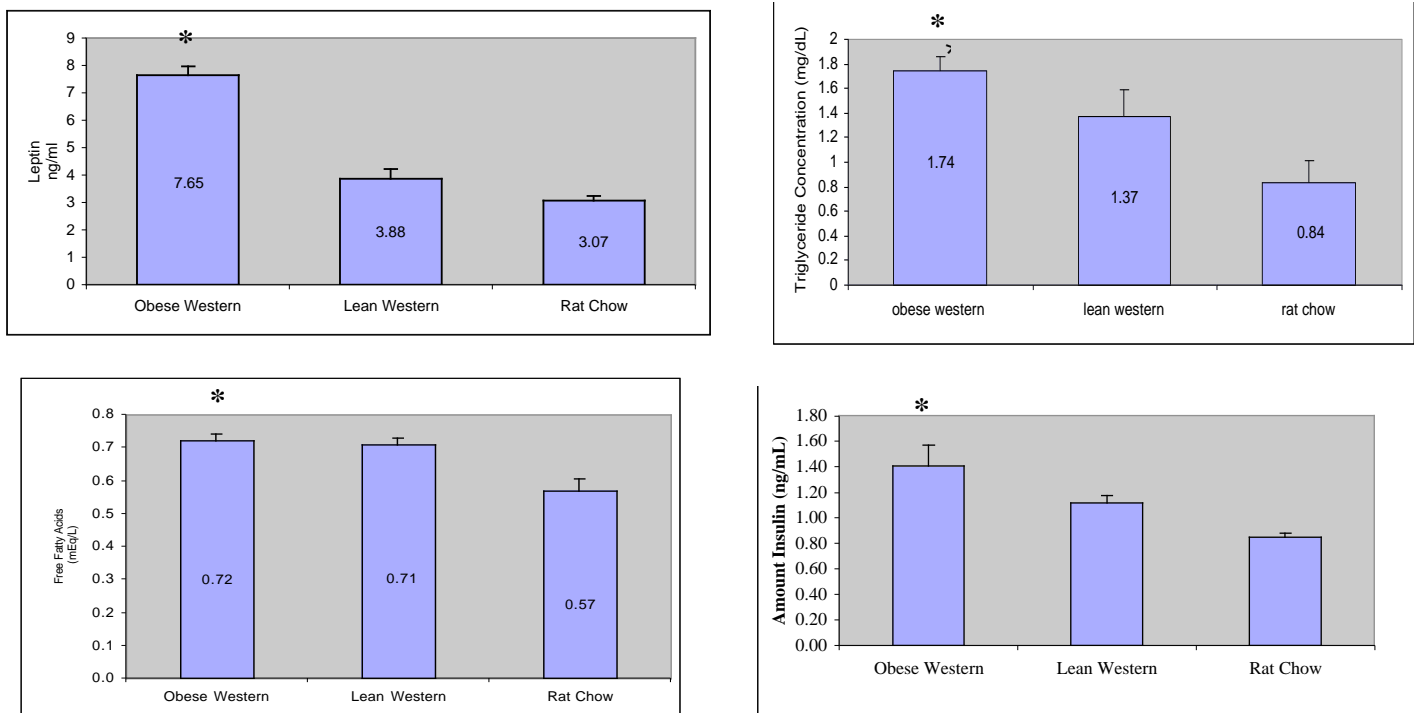
Figure 3. Serum Comorbidity factors in Obese and Lean Western diet and Lean Rat Chow diet rats. Female rats were weaned at 21 days old and randomly placed on either a Western Diet (Diet Research) or a Rat Chow diet (LabDiet). At 25 weeks DEXA Scans were performed to determine body fat composition and define all three groups. Rats with the highest body fat mass were assigned to the Obese Western (DIO) group, (n=13). Rats with the lowest fat mass were designated Lean Western (n=12). Rat Chow fed animals had an n=15. Immediately after scanning rats were sacrificed and blood serum was isolated. Serum Leptin and Insulin levels were measured via RIA kit (Linco). Serum free fatty acids and triglycerides were measured via colorimetric assay (Sigma). Data represents means $\pm$ SE. * = p 0.05 or less.

Onset of puberty in DIO rats

In humans it is well established that the onset of puberty is dependent in part on the accumulation of body fat. A classic example is the delayed menarche seen in lean young women that are long distance runners (8). Over the past century the average age at menarche of American women has decreased from 14.2 yrs to 12.5 yrs (9). This secular trend has been attributed to improved environmental conditions, improved nutrition and obesity. With these evidences in mind, we hypothesized that in our rat DIO model the Western Diet fed animals would undergo puberty at an earlier age compared to Rat Chow fed Controls. We tested this hypothesis by evaluating vaginal introitus (vaginal opening), a commonly used indicator of estrus cycle onset in the rodents described above (10). In Figure 4 we show that at 43 days of age, 100% of the Western Diet fed rats and only 60% of the Rat Chow fed animals showed vaginal opening. Not until day 53 did 100% of Rat Chow fed controls exhibit vaginal opening. These results indicate that, compared to Rat Chow fed controls, our Western diet induces an earlier onset of puberty in young female Sprague-Dawley rats. This model follows the same trend in humans where childhood obesity leads to an earlier age of menarche in females.
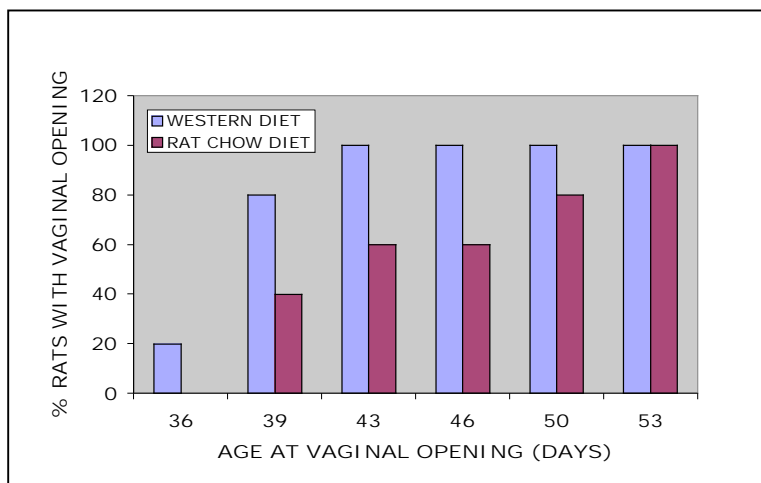
Mammary Tumor incidence in Western diet fed rats

In a subsequent study we evaluated the onset of MNU-induced mammary tumors in our rat model of DIO. As described above we produced Obese Western (n=13), Lean Western (n=13) and Lean Rat Chow (n=13) SD female rats and at 65 days old each group was injected with Methylnitrosourea (MNU). The development of mammary tumors via MNU treatment is a widely accepted model of carcinogenesis (11). Recent molecular profiling studies have revealed that MNU-induced rat tumors are similar to low-grade human mammary tumors and are an excellent model for breast cancer (12). Another major advantage of this system is that hormone dependent tumors are produced which can progress to the hormone independent state, as is common in human breast cancer (11). After injection the three groups of rats were palpated twice a week and tumor load and latency period were recorded. When comparing the three groups, significant differences in tumor onset were observed after 25 weeks post MNU injection. Briefly, mammary tumors appeared sooner and in greater numbers in Obese and Lean Western fed rats as compared to Chow fed Lean rats (Figure 5 and Table 3). Histological analysis of tumors revealed the Obese group had the highest percentage of hi-grade mammary carcinoma tumors (92% of total tumor number) as compared to the other two groups (50%) (Table 3 and Figure 6). These data demonstrate that mammary tumorigenesis is more aggressive in Obese compared to Lean rats. Also we delineate that consumption of a Western diet, in the absence of obesity, may lead to a sooner onset of mammary tumor formation.
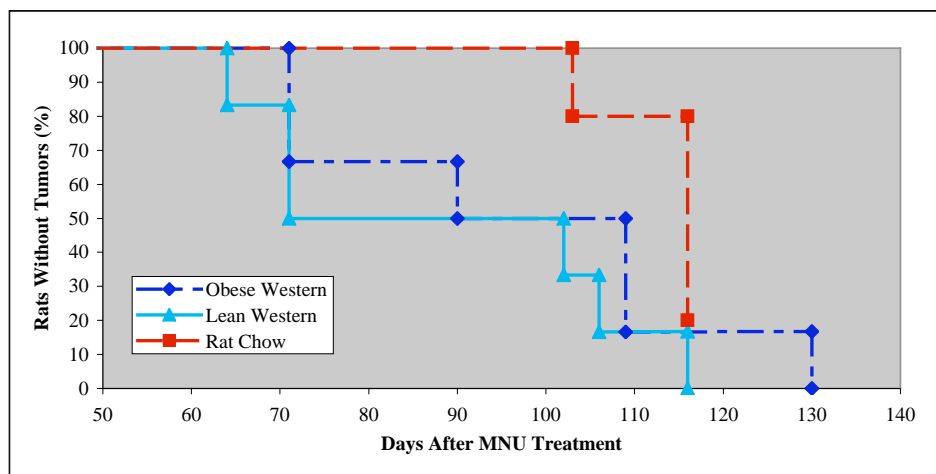

Figure 5. Influence of Obesity and Western Diet on mammary tumorigenesis in Sprague-Dawley Rats. Rats were placed on Western or Rat Chow Diets as described previously. Based on % body fat mass (DEXA scan at 54 days) rats were placed into three groups; Western Diet Obese (n=13), Lean Western (n=13) and Lean Rat Chow controls (n=13). At 64 days of age animals in each group were anesthetized and given a single intraparitoneal injection of MNU (50mg MNU/kg body weight). All rats were palpated twice a week to detect mammary tumors. Mammary tumor latency period and load are presented in graph above and table form below.

Table 3. Mammary tumor development in Sprague Dawley rats 25wks after MNU injection

Diet	Group	# Rats	Detection of First Tumor (Day)	Total Number of Tumors	Histology (# of Hi grade carcinomas: # of adenomas*)	Hi grade Carcinomas (% per total # of tumors)
Western	Obese	13	71	24	22:2	92%
Western	Lean	13	74	18	9:6	50%
Rat Chow	Lean	13	109	14	7:3	50%

*Remainder of tumors in each group were Lo grade carcinomas.

Obese Adenocarcinoma   **Lean Rat Chow Adenoma**   **Rat Chow Normal gland**
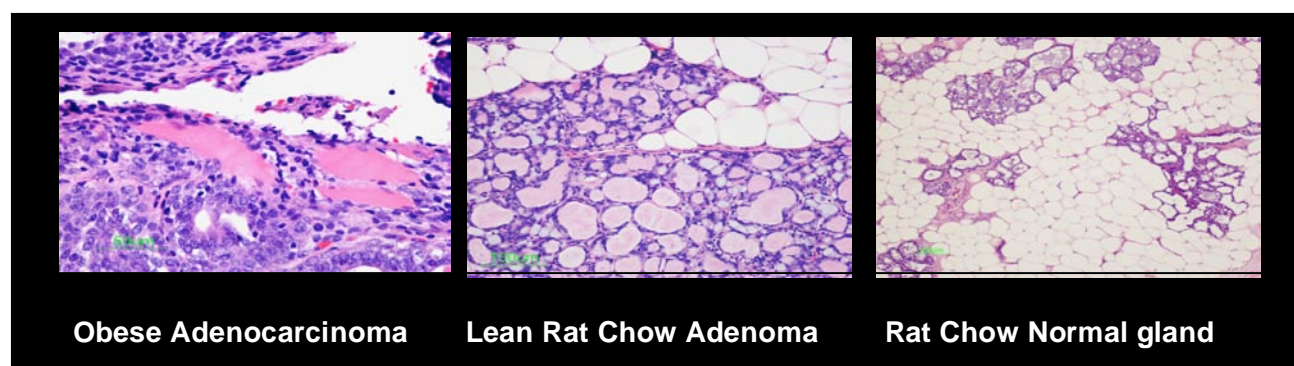
Figure 6. Influence of Obesity and Western Diet on mammary tumor grade in Sprague-Dawley Rats. As described above, three groups of rats were produced; Western Diet Obese, Lean Western and Lean Rat Chow controls. Animals in each group were given a single injection of MNU (50mg MNU/kg body weight). At 25 wks tumors and mammary tissue were collected and prepared for histological analysis via hematoxylin and eosin staining. Predominantly, more aggressive mammary tumors appeared in the Obese group. The left panel shows an adenocarcinoma from an Obese rat where tumor cells (purple) are invading adjacent muscle tissue (pink). In the middle panel a benign adenoma, with adjacent fat cells (white), from a Lean rat is shown. On the right is a tumor-free gland from a lean rat. This gland consists primarily of fat cells with a few primary epithelial ducts (purple).

Evaluating the influence of Obesity on the Mammary Gland and Mammary Tumor Stroma

The goal of this set of studies was to support our *in vitro* leptin-MCF-7 mammary tumor cell findings with our new *in vivo* model of obesity and breast cancer, with a focus on collagen expression (please see Appendix section for the abstract "Role of Childhood Obesity and Leptin in Breast Cancer", from 2006 American Cancer Society Conference invited talk). Briefly, mammary glands and tumors were analyzed from control and MNU treated Western Obese, Lean Western and Lean Rat Chow animals from the study described above. The Obese group had the highest numbers of tumors that were predominantly of the aggressive phenotype, histologically. As increasing evidence indicates microenvironment affects tumorigenesis, we analyzed rat mammary tissues and tumors with new nonlinear optical imaging technologies. We are the first to use Coherent Anti-Stokes Raman Scattering and Second Harmonic Generation on the same platform to simultaneously image tumor cells, adipocytes and collagen fibrils, a technique not requiring tissue labeling. Multiphoton imaging of mammary stroma revealed a significant impact of diet and obesity on adipocytes and collagen levels. Adipocyte size increased in Obese and Lean rats on a Western Diet compared to Chow fed rats, suggesting that increased fat mass of Obese rats is associated with adipocyte proliferation. Furthermore, increased collagen production was observed in Obese rats and was positively associated with tumor aggressiveness. These results provide excellent evidence that the leptin regulation of collagen, demonstrated *in vitro*, may be an important mechanism towards tumor growth and aggressiveness *in vivo*. In our initial *in vivo* goals we proposed to use LCM coupled with gene array methods to measure leptin-regulated gene expression in mammary tumor cells from obese rats. As we engaged these studies, our interest focused heavily on measuring collagen expression. This focus was further stimulated by a collaboration where collagen could be easily and rapidly measured in our tissues (CARS microscopy, performed by Dr. Ji-Xing Cheng's group.) This *in vivo* work has allowed us to complete one manuscript that has recently been published in *Molecular Imaging* (Please see the publication "Nonlinear optical imaging to evaluate the impact of Obesity on Mammary gland and Tumor Stroma" provided I the

appendix). This manuscript describes the evaluation of collagen, tumor cells and adipocytes of animals from our new model of obesity and breast cancer. This further characterization of our rat model has provided a foundation of data that has been important for attracting funding from several sources including a USDA training grant, an NSF Graduate Fellowship, a Komen Breast Cancer Foundation grant and an NCI grant. Furthermore we have received excellent recognition for our studies, as our work was selected as one of the most newsworthy abstracts presented at the 2006 National Endocrine Society Conference. We have also been invited to present our work at subsequent Endocrine Society and American Cancer Society conferences and have received travel awards in connection with these presentations (please see *Reportable Outcomes* section).

Key Research Accomplishments

Model of Childhood Obesity
- We have developed and characterized a new female rat model of Childhood onset Diet Induced Obesity (DIO).
- Using outbred Sprague Dawley rats we have shown that, compared to Rat Chow, consumption of a Western diet caused increased body fat mass in approximately 20-30% of animals within 54 days. Obese rats had a 2-fold higher % fat mass as compared to Chow Fed rats.
- We demonstrate that the 2-fold difference in % fat mass between the Obese Western and Lean Rat Chow animals was maintained through 25 weeks.
- Animals on Rat Chow consumed more food in *grams* per day, as compared to Western Diet fed rats. However, Rat Chow and Western fed groups had the same caloric intake per day over 25 weeks.
- Vaginal opening, and indicator of puberty onset, occurred earlier in the Western Diet group as compared to Rat Chow fed animals.
- As in human obesity, we showed that Obese Western rats had 2-3 fold higher serum leptin and higher TG, FFA and insulin levels, compared to Lean Rat Chow animals.

Mammary Tumorigenesis in Early Onset Obese Rats
- We demonstrate that in Western Diet fed rats, MNU-induced mammary tumors appear sooner as compared to Rat Chow animals.
- We have shown that Obese Western rats had greater numbers of mammary tumors than Lean Western and Lean Rat Chow animals.
- Based on histological classification, we demonstrate that Obese Western animals have predominately hi-grade invasive type mammary tumors compared to Lean rats on the Western or Rat Chow diets.
- Adipocyte size increased in Obese and Lean rats on a Western Diet compared to Chow fed rats, suggesting that adipocyte proliferation is associated with increased fat mass of Obese rats.
- Increased collagen levels were observed in Obese rats and was positively associated with tumor aggressiveness.
- Rat DIO model studies support that the leptin regulation of collagen, demonstrated *in vitro*, may be an important mechanism towards tumor growth and aggressiveness *in vivo*.

Unexpected delays in proposed work/remaining Task 2

The above accomplishments represent the majority of Task 1 as outlined in our grant proposal. We did experience unforeseen delays in beginning our work, as there was problems with DEXA scan equipment malfunctions. Furthermore, there were restrictions regarding the number of animals that could be scanned within a given number of days. For the latter reason we were forced to break up our study into two repeats with less animals per repeat (instead of performing our entire study at all at once). We have completed all animal studies and have collected all necessary tissues to complete our project. To complete Task 1 we are currently determining the estrogen receptor status of mammary tumors from DIO and Lean control rats by immunocytochemistry (IHC). We have experienced extensive delays in our IHC methodologies as tissues were inadvertently over fixed in formalin. We have been working with a board certified pathologist and have recently been successful in develop working unmasking protocols to stain our samples. Simultaneously, towards Task 2 we have prepared mammary tissues and tumors for laser capture microdissection. As our efforts have been heavily focused on development of IHC protocols, we are still working towards epithelial and adipocyte cell specific RNA isolation and Affymetrix array gene chip analyses. The IHC protocols were prioritized as this information would allow us to complete the next obesity rat model manuscript.

Reportable Outcomes

Presentations, Abstracts and Publications

I.G. Camarillo, C. Rehrer, Chris Gottfried and M. Nichols. "Mammary Tumorigenesis in a Novel Rat Model of Childhood Onset Diet-Induced Obesity"" Endocrine Society 88th Annual Conference, Boston MA, 2006. *- Abstract selected for Endocrine Society Conference Research Summaries Book; A listing of 100 newsworthy abstracts- over 3,000 abstracts submitted. Professor Camarillo also received a FASEB MARC Travel award to present this research.*

"The influence of Childhood Onset Obesity on Breast Cancer; A new animal model" Purdue University Oncological Sciences Center, Progress and Problems in Cancer Prevention and Control symposium. Purdue University, W. Lafayette, January 17, 2006. *Invited talk.*

C. Rehrer, Chris Gottfried, M. Nichols and I.G. Camarillo. "A Female Rat Model of Childhood Onset Diet Induced Obesity" Peachy Breast Cancer Conference, Indiana University-Purdue University, Indianapolis, Indiana, February 9, 2006.

Purdue Cancer Center-Director's Advisory Board Meeting, West Lafayette, IN, September 29, 2006. "Obesity, Leptin and Breast Cancer".- *Featured Speaker.*

Camarillo, I. G., Perera, C.N., Rehrer C., Nichols M., Le. T. and J. Cheng. " The role of Childhood Obesity and Leptin in Breast Cancer" 14th Annual American Cancer Society Great Lakes Cancer Symposium Van Andel Research Institute, Grand Rapids, MI, October 16, 2006. *–Invited Speaker- This Abstract is included in the Appendix section of this report.*

"Multiphoton imaging to determine the influence of diet and obesity on mammary tumorigenesis" Society for Advancement of Chicanos and Native Americans in Science (SACNAS) National Conference, Tampa, Florida, October 26-29, 2006.

"The role of obesity and leptin in breast cancer," Department of Biology – Division of Life Sciences, University of Texas at San Antonio, San Antonio, Texas, April 27, 2007.

Rehrer, C., T. Le, C. Gottfried, M. Nichols, J. Cheng and Ignacio Camarillo. "Non Linear Optical Imaging to Determine the Influence of Obesity on Mammary Microenvironment and Tumorigenesis" Endocrine Society 89th Annual Conference, Toronto, Canada, June 2-5, 2007. *Endocrine Society Travel Award Winner- This Abstract is included in the Appendix section of this report.*

Reportable Outcomes, Continued

T. Le, C. Rehrer, B. Huff, M. Nichols, I.G. Camarillo and J. Cheng. Nonlinear optical imaging to evaluate the impact of Obesity on Mammary gland and Tumor Stroma. *Molecular Imaging* 2007 Jul-Sep;6(3):205-11.

Awards/Recognitions as a result of this grant

Endocrine Society Conference Research Summaries Book (newsworthy abstracts) 2006
FASEB MARC Travel Award, 2006
AACR Minority Scholar Award, 2006
Charles Rehrer, National Science Foundation Graduate Research Fellowship, 2006
Charles Rehrer**,** Graduate Student Fellowship Incentive Award, 2006
Hwei-Gene Chin (undergrad), Purdue/Howard Hughes Medical Institute Summer Internship, 2006
Julie Wilmowski (undergrad), Purdue/Howard Hughes Medical Institute Summer Internship, 2006
FASEB MARC Travel Award, 2007
Endocrine Society Travel Award, 2007
USDA National Needs fellowship training grant, Nutrition & Disease, 2007

Conclusion

Obesity is a major health problem in the U.S. and is associated with increased breast cancer incidence and mortality. The epidemic of childhood obesity is recent and little information exists regarding its association with breast cancer. Towards better understanding this relationship we have developed and characterized a new rat model of childhood onset Diet Induced Obesity (DIO) and breast cancer. We have shown that 20-30% of young female ourtbred rats on a Western diet have higher body fat mass and disregulated serum comorbidity factors, as compared to Chow fed Lean rats. This obese rat model better parallels the onset of obesity as it occurs in humans. We have shown that in Western Diet fed animals (Obese *and* Lean), MNU-induced mammary tumors appear sooner as compared to Rat Chow fed Lean rats. Furthermore, Obese rats had higher numbers of tumors than Chow fed rats. Via histology we determined that mammary tumors from Obese rats are predominately of an invasive phenotype, compared to tumors from Lean rats. This aggressive phenotype is in accord with the association between human obesity and breast cancer mortality. Interestingly, this work provides new information suggesting that consumption of a western diet by lean individuals may affect breast cancer risk. We also provide evidence that a Western diet in the context of obesity can influence tumor load and phenotype. As a scientific product, this new model provides an excellent system to study the underlying mechanisms of childhood obesity and mammary tumor formation and progression. Future studies will exploit this model to better understand adipocyte-epithelial interactions during mammary tumorigenesis, identify and validate novel molecular therapeutic targets, and to establish biomarkers and imaging technologies for cancer prevention and prognosis.

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APPENDIX

The Role of Childhood Obesity and Leptin in Breast Cancer

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Obesity is a risk factor for breast cancer incidence and mortality. The recent epidemic of childhood obesity emphasizes a need to define the relationship between early onset obesity and breast cancer. Towards this end, we developed a unique childhood Diet Induced Obese (DIO) model of breast cancer which mimics the progression of human breast cancer. We have exploited this model to determine the influence of obesity on mammary tumor onset, phenotype and microenvironment architecture. In addition, we have employed proteomic and genomic approaches to gain mechanistic insights into the role of the obesity hormone leptin in MCF-7 human breast cancer cells.

In our animal model of obesity a "Western Diet" high in fat and simple carbohydrates was used, representing the typical human diet of North America. Briefly, at weaning outbred Sprague Dawley rats were randomly placed on either a Western or Rat Chow control diet for five weeks, then DEXA scanned to determine percent body fat mass. Rats were then divided into three groups; Western Obese, Lean Diet Resistant Western and Lean Rat Chow. Western Obese animals exhibited a significant 2-fold increase in fat mass as compared to the other two groups. No significant differences in net assimilated energy consumption existed among groups, indicating that hyperphagia was not responsible for fat mass differences. Western Obese rats had elevated serum comorbidity factors insulin, leptin, plasma glucose, free fatty acids and triglycerides as compared to Lean Rat Chow Controls. When each group was treated with the chemical carcinogen Methylnitrosourea (MNU), mammary tumors appeared sooner in all rats on the "Western Diet", compared to Chow fed Lean rats, and the Obese group had the highest numbers of tumors. Histological classification showed a predominantly aggressive phenotype in tumors from the Western Obese group. This finding is in accord with the association between obesity and morbidity in human breast cancer. As increasing evidence indicates tissue microenvironment affects tumorigenesis, we analyzed rat mammary tissues and tumors with new nonlinear optical imaging technologies. We are the first to use Coherent Anti-Stokes Raman Scattering and Second Harmonic Generation on the same platform to simultaneously image tumor cells, adipocytes and collagen fibrils, a technique not requiring tissue labeling. Multiphoton imaging of mammary stroma revealed a significant impact of diet and obesity on adipocyte and collagen 3-D structure, parameters not accessible by standard two-dimensional histological evaluation. Adipocyte size increased in Obese and Lean rats on a Western Diet compared to Chow fed rats, suggesting that increased fat mass of Obese rats is associated with adipocyte proliferation. Furthermore, increased collagen production was observed in Obese rats and was positively associated with tumor aggressiveness. These studies provide new insights into the relationship between obesity and breast cancer.

A characteristic of obesity is elevated circulating levels of leptin, an adipocyte-derived hormone that stimulates mammary tumor cell growth *in vitro*. Towards determining the mechanisms of leptin we used proteomic and gene array methods to identify leptin-regulated molecules in MCF-7 human breast cancer cells. We have shown leptin induces proliferation and regulates the secretion of several proteins from MCF-7s including collagen, TGF- ß3 and IGFBP-3. By microarray we identified more than 25 leptin-regulated genes that included growth factor, extracellular matrix, cell cycle and metastasis genes. Uncovering these distinct signaling pathways and autocrine/paracrine factors regulated by leptin provide valuable clues for determining the relationship between leptin and tumor progression *in vivo*.

In summary, our results demonstrate consumption of a Western style diet at an early age has a significant impact on comorbidity factors, mammary tumorigenesis and mammary architecture. We also provide *in vitro* evidence that leptin affects mammary tumor cells by regulating mitogenic cell signaling pathways, autocrine/paracrine growth factors and by modifying extracellular matrix composition. Identification of leptin-regulated factors provides mechanistic links into the relationship between obesity and increased breast cancer incidence and morbidity. Our approach using our unique animal model of obesity, human MCF-7 breast cancer cells, and novel imaging technologies represent a valuable system to better understand adipocyte-epithelial interactions during mammary tumorigenesis, establish biomarkers for cancer prevention and prognosis, and to identify and validate new molecular therapeutic targets for obesity-associated cancer.

Use of Non Linear Optical Imaging to Determine the Influence of Childhood Onset Obesity on Mammary Microenvironment and Tumorigenesis

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Obesity is a risk factor in the incidence of many cancers, including breast cancer. The epidemic of childhood obesity emphasizes the need to determine the relationship between early onset obesity and breast cancer. Towards this end, we have developed a unique childhood onset Diet Induced Obese (DIO) model of breast cancer that parallels the progression of obesity and breast cancer in humans. Briefly, weaning Sprague-Dawley rats were placed on either a Western style or Rat Chow Diet. After five weeks animals were grouped into Western Obese, Western Diet Resistant Lean and Rat Chow Lean, based on percent body fat mass, and injected with MNU to induce mammary tumors. We have exploited this model to determine the influence of obesity on mammary microenvironment architecture, tumor progression, and phenotype.

As increasing evidence indicates tissue microenvironment significantly impacts tumorigenesis, we analyzed rat mammary tissues and tumors with new nonlinear optical imaging technologies. We are the first to use Coherent Anti-Stokes Raman Scattering and Second Harmonic Generation on the same platform to simultaneously image tumor cells, adipocytes and collagen fibrils in 3 dimensions, a technique not requiring tissue labeling. Multiphoton imaging of stroma revealed a significant impact of diet and obesity on mammary adipocytes and collagen content,. Adipocyte size increased in Obese and Lean rats on a Western Diet as compared to Chow fed rats, suggesting that the increased fat mass of Obese rats is associated with adipocyte proliferation. Furthermore, increased collagen content was observed in tumors from Obese rats, compared to Western or Rat Chow Lean rats, and was positively associated with tumor aggressiveness.

Our results demonstrate consumption of a Western style diet at an early age induces significant changes in mammary microenvironment architecture, tumor progression, and phenotype. This unique model of early onset obesity and breast cancer represents a valuable tool for identifying and validating novel molecular targets for obesity-associated cancer therapies.

Nonlinear Optical Imaging to Evaluate the Impact of Obesity on Mammary Gland and Tumor Stroma

Thuc T. Le, Charles W. Rehrer, Terry B. Huff, Maxine B. Nichols, Ignacio G. Camarillo, and Ji-Xin Cheng

Abstract

Obesity is an established risk factor for breast cancer incidence and mortality. However, the mechanism that links obesity to tumorigenesis is not well understood. Here we combined nonlinear optical imaging technologies with an early-onset diet-induced obesity breast cancer animal model to evaluate the impact of obesity on the composition of mammary gland and tumor stroma. Using coherent anti-Stokes Raman scattering and second harmonic generation on the same platform, we simultaneously imaged mammary adipocytes, blood capillaries, collagen fibrils, and tumor cells without any labeling. We observed that obesity increases the size of lipid droplets of adipocytes in mammary gland and collagen content in mammary tumor stroma, respectively. Such impacts of obesity on mammary gland and tumor stroma could not be analyzed using standard two-dimensional histologic evaluation. Given the importance of mammary stroma to the growth and migration of tumor cells, our observation provides the first imaging evidence that supports the relationship between obesity and breast cancer risk.

O BESITY is an established risk factor for a number of human diseases, such as diabetes, heart disease, and breast cancer.[1,2] A recent rapid increase in childhood obesity indicates that the adverse effects of obesity on human health will be a major concern for the near future. In particular, the risk of breast cancer increases significantly with an elevated body mass index (> 30 kg/m^2).[2,3] Despite a well-established association, the relationship between obesity and breast cancer is not well understood. A common approach to studying breast cancer is to investigate mutations of oncogenes.[4] However, the process in which a normal cell transforms into cancer is driven not only by cellular intrinsic events, such as genetic mutations, but also by extrinsic factors in the microenvironment.[5] Phenotypic behaviors of cancer cells, such as proliferation and invasion, are regulated by extracellular matrix (ECM) components and soluble secreted factors of surrounding stromal cells.[5–7] The dynamic interactions of cancer cells and the local environment play crucial roles in their survival and development into malignancy.[6,8] Disrupting these interactions has become a viable therapeutic strategy for cancer treatment in recent years.[9,10]

To further understand the influence of the microenvironment on mammary tumorigenesis, we used nonlinear optical (NLO) imaging to characterize the stroma of mammary gland and tumor tissues. Although histologic studies enable the analysis of thin slices of tissue biopsy, they lack three-dimensional information crucial to describe stromal organization in their natural state. On the other hand, NLO microscopy has demonstrated successfully the ability for deep tissue imaging with three-dimensional resolution.[11] Using second harmonic generation (SHG) and two-photon excitation fluorescence (TPEF) microscopy, a number of research groups have imaged collagen type I, tumor cells expressing green fluorescent protein, and dye-labeled vasculatures in living animals.[12–16] For molecules that cannot tolerate fluorophore labeling, coherent anti-Stokes Raman scattering (CARS) microscopy provides a highly sensitive vibrational imaging technique.[17] Recently, CARS has been successfully applied to image lipid domains, cell membranes, axonal myelin sheath in live tissues, and adipocytes in live animals.[18–21] Here we combine CARS, SHG, and TPEF microscopy into a multimodal platform to image components of the mammary stroma, such as adipocytes, collagen fibrils, blood vessels, and others.

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Specifically, we asked how obesity impacts the composition of mammary gland and tumor stroma, leading to the observed relationship between obesity and high breast cancer risk.[3,22] To address this question, we used a Sprague-Dawley rat model of early-onset diet-induced obesity (DIO; see Methods; Figure S1*).[23,24] DIO animal models have been used to study many diseases associated with obesity.[25] We first placed a group of young rats on a high-fat Western diet to induce obesity and another group on a lean rat chow diet to serve as controls. Then we treated one group of rats with methylnitrosourea (MNU), a chemical carcinogen that induces mammary tumors, and the other group of control rats with saline.[26] At specified times (see Methods), we collected mammary gland and tumor tissues for NLO imaging (Figure S2). Based on CARS imaging of adipocytes and SHG imaging of collagen fibrils, we observed that obesity increases the size of lipid droplets of adipocytes in the mammary gland and collagen content in mammary tumor stroma. The impact of obesity on adipogenesis and collagen content should further our understanding of an established but mechanistically undefined role of obesity on breast cancer incidence and mortality.

Materials and Methods

Animal Model

Of the 70 outbred Sprague-Dawley rats at 21 days old, 19 rats were placed on a rat chow diet (Harlan Tech, Indianapolis, IN) and 51 rats on a Western diet (Research Diets, New Brunswick, NJ) to produce 19 lean rat chow and 17 DIO Western animals (see Figure S1). The rat chow diet contained 3.30 kcal/g and the Western diet contained 4.47 kcal/g of metabolizable energy. No significant difference was found in kcal/g consumption among groups (data not shown). At 54 days old, rats were grouped according to their body fat composition determined by dual-energy x-ray absorptometry (DEXA; Lunar Corporation, Madison, WI) under isoflurane anesthesia. The average percent body fat mass of rats on the lean rat chow diet was half of that on the obese Western diet. At 65 days old, half of the rats in each group received the carcinogen MNU and the other half a 0.9% saline injection. The resulting four groups of animals comprised 10 lean rat chow and 8 obese Western rats with no tumor and 9 lean rat chow tumor and 9 obese Western tumor rats. At 182 days old, the body fat

composition of the rats was again determined by DEXA scans. The average percent body fat mass for lean rat chow rats was approximately half of that of the obese Western rats (data not shown). Rats were sacrificed either at 233 days old or when the animals reached physiologic and ethologic end points of pain, distress, and suffering as determined by established guidelines.[27] Mammary gland and tumor tissues of three rats from each animal group were collected and stored for multiphoton imaging or prepared for histologic evaluation. All animal experiments were done with the approval of the Purdue Animal Care and Use Committee.

Tissue Maintenance

Mammary gland and tumor tissues were cut into small 2 × 2 × 2 mm pieces and kept in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 37°C incubator with 5% CO_2. Mammary gland tissues were imaged within 7 days after collection. Mammary tumor tissues were either kept in EMEM for imaging within 24 hours after tissue collection or kept frozen in liquid nitrogen for imaging at a later time (Figure S3).

Histology Preparation

Mammary gland and tumor tissues were fixed in a solution of 10% buffered formalin. After fixation, tissues were processed and embedded in paraffin blocks. Tissue sections of 5 μm thickness were prepared and stained with hematoxylin-eosin.

Nonlinear Optical Imaging

A multimodal NLO microscope that allows CARS, SHG, and TPEF imaging on the same platform is diagrammed in Figure S2. For CARS imaging, two tightly synchronized Ti: Sapphire lasers (Mira 900, Coherent Inc., Santa Clara, CA) with an average timing jitter of 100 fs were used. Both lasers have a pulse duration of 2.5 ps and operate at a 78 MHz repetition rate. The two beams at frequencies of ω_p (pump) and ω_s (Stokes) were parallel polarized and collinearly combined. A Pockels' cell (Model 350-160, Conoptics, Danbury, CT) was used to reduce the repetition rate to ≈4 MHz. The combined beams were directed into a laser scanning microscope (FV300/IX70, Olympus, Melville, NY) and focused into a sample through a 60× water immersion microscope objective (numerical aperture = 1.2). Back-reflected signal was collected by the same objective, spectrally separated from the excitation source by a dichroic mirror (670dcxr, Chroma Technologies, Rockingham, VT), trans-

mitted through a 600/65 nm bandpass filter (42-7336, Ealing Catalog Inc., Rocklin, CA), and detected by a photomultiplier tube (PMT; H7422-40, Hamamatsu, Japan) mounted at the backport of the microscope. We tuned the pump laser (ω_p) to around 14,240 cm^{-1} and the Stokes laser (ω_s) to around 11,400 cm^{-1}. Their wave number difference $\omega_p - \omega_s$ = 2,840 cm^{-1} matches the Raman shift of symmetric CH_2 stretch vibration. For SHG and TPEF imaging, a femtosecond laser (795 nm, 200 fs) at 78 MHz (Mira 900, Coherent Inc.) was used for excitation. SHG and TPEF signals were detected by the same backport-mounted detector as CARS signal. Bandpass filters 375/50 nm and 520/40 nm (Chroma Technologies) were used to transmit SHG and TPEF signals, respectively. The TPEF filter 520/40 nm was selected specifically for fluorescein dye. The total acquisition time for each image was 1.12 seconds. Images were analyzed using *FluoView* software (Olympus, Melville, NY).

Imaging Condition

A glass-bottom chamber containing tissues submerged in maintenance media was placed on the microscope for imaging at room temperature. For CARS imaging, the total power of Stokes and pump lasers at the sample was set at 4 mW. For SHG imaging of fresh tissues and histology tissue sections, the laser power was set at 40 mW and 16 mW at 795 nm, respectively. For TPEF imaging, laser power was set at 16 mW at 795 nm.

Evaluating Mammary Stromal Collagen Content

An analysis volume is defined with the dimensions of 250 μm (x) × 250 μm (y) × 40 μm (z), with z = 0 being the coverslip and tissue interface (Figure S4). For each analysis volume, 81 frames along the z-axis with a fixed step size of 0.5 μm were acquired. Nine different volumes were evaluated, and the total SHG intensity (minus the background intensity) from 729 frames (9 × 81 frames) was used to infer the mammary stromal collagen content of each animal. Background intensity was collected from an analysis volume devoid of any collagen. The laser power at the sample and the high voltage of the PMT was kept constant for all measurements.

Measuring the Diameters of Lipid Droplets of Adipocytes

Adipocytes within the mammary gland tissues of saline-treated rats were scanned along the z-axis with a 0.5 μm step size using CARS microscopy. The largest measured diameter values of lipid droplets at equatorial sections

were recorded. One hundred adipocytes in the mammary stroma of each rat were evaluated to obtain the average lipid droplet diameter (Table S1).

Results

Using CARS and SHG on the same microscope platform (see Figure S2), we imaged the composition and structural organization of mammary gland and tumor stroma. We found that multimodal NLO imaging enables simultaneous visualization of adipocytes, collagen fibrils, and tubular structures representing blood capillaries (Figure 1A, supplementary movie 1*). Because imaging blood capillaries without labeling has not been reported previously, we labeled the endothelial cells lining the blood vessels with fluorescein isothiocyanate conjugated isolectin B$_4$ (FITC-IB$_4$).[28,29] The TPEF image in Figure 1B exhibited strong FITC-IB$_4$ staining of tubular structures, as well as activated macrophages, in the mammary gland (supplementary movie 2).[29] We observed that most adipocytes were surrounded by collagen fibrils in the mammary gland (Figure 1C, supplementary movie 3). In addition, we showed that CARS enabled imaging of tumor cells in mammary tumors without any labeling (Figure 1, D–F). Tumor cell visualization was possible owing to weak CARS signals arising from the cell membrane's lipid bilayer and strong CARS signals from numerous small lipid droplets surrounding the nucleus, which gives a dark contrast in the CARS image (supplementary movie 4). Tumor cell visualization was further confirmed by DiOC18 fluorescence imaging (see Figure S4).[30] In contrast to the orientation of collagen fibrils around adipocytes in the mammary gland, we found that most collagen fibrils were located at the outer perimeter of the tumor mass in mammary tumor (see Figures 1, D–F, and S5). This spatial organization of collagen relative to tumor mass is consistent with a previous report by Ahmed and colleagues in which SHG and TPEF microscopy were used to image the organization of collagen and mammary tumor cells expressing green fluorescent protein in transgenic mice, respectively.[13] However, the use of CARS microscopy to image tumor cells does not require any labeling and should be applicable to image any type of tumor.

With the demonstrated capability of CARS and SHG for label-free imaging of adipocytes and collagen, respectively, we proceeded to evaluate the impact of obesity on their composition in mammary gland and tumor stroma. We

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*The supplementary movies can be viewed online only by members and subscribers at <www.bcdecker.com>.
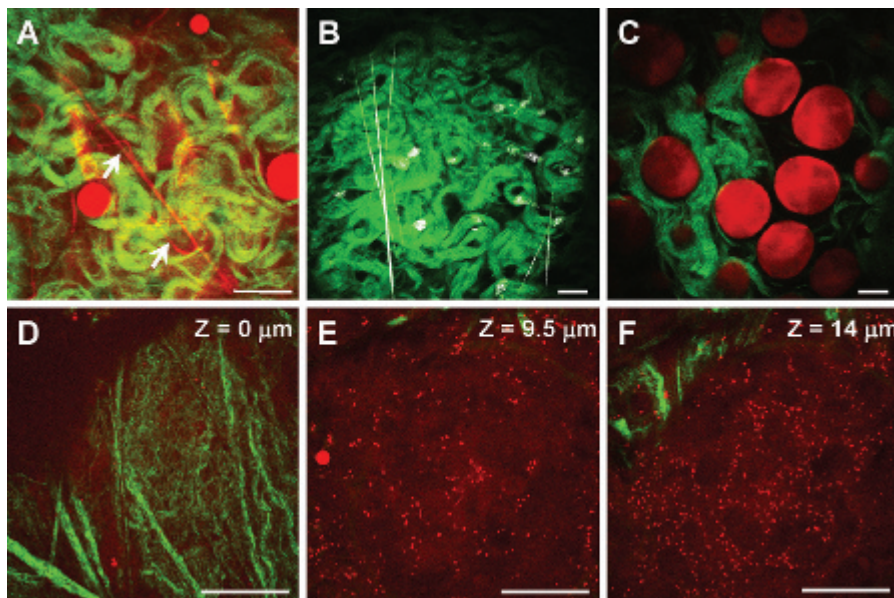
Figure 1. Nonlinear optical imaging of mammary gland and tumor stromal composition. *A*, Adipocytes and blood capillaries (*arrows*) imaged with coherent anti-Stokes Raman scattering (*red*) and collagen fibrils imaged with second harmonic generation (SHG) (*green*) in a mammary gland. *B*, Blood capillaries and macrophages stained with fluorescein isothiocyanate conjugated isolectin B_4 and imaged with two-photon excitation fluorescence (TPEF) (*gray*). Collagen fibrils was imaged with SHG (*green*). *C*, Adipocyte (*red*) and collagen fibrils (*green*) organization in a mammary gland. *D–F*, Organization of tumor cells (*red*) and collagen fibrils (*green*) along the vertical axis of a mammary tumor stroma. Images taken with a 60× water immersion objective. Scale bars = 25 μm.

imaged mammary tissues collected from three rats for each of the four experimental groups: lean rat chow, obese Western, lean rat chow tumor, and obese Western tumor. Because SHG intensity is directly correlated with the concentration of collagen fibril type I, we evaluated the relative collagen content of a tissue based on total SHG intensity.[14] We found that there were strong correlations between obesity, ECM collagen content, and adipocyte size. In the mammary gland, we observed higher collagen content in lean rats on the rat chow diet compared with obese rats on the Western diet (Figure 2, A and E). Conversely, in mammary tumor stroma, we observed higher collagen content in obese rats on the Western diet compared with lean rats on the rat chow diet (Figure 2, B–D and F–H). To systematically compare the ECM collagen content from one animal with that of the other, we defined a fixed analysis volume and collected total SHG signals from nine volumes in the mammary gland or tumor stroma of each rat (see Methods; Figure S6). The total SHG collagen intensity as a function of rats in different diet groups is plotted in Figure 3A (see Table S1). Consistent with our observation, quantitative analysis of SHG intensity indicated that obesity decreases collagen content by an average of 5-fold in the mammary gland and increases collagen content by an average of 14-fold in mammary tumor stroma.

To analyze the impact of obesity on adipogenesis in mammary stroma, we used CARS to evaluate the size of lipid droplets in adipocytes. By focusing CARS excitation beams at the equatorial planes, we measured the diameters of 100 lipid droplets of adipocytes in each rat in both diet groups (see Table S1). The average lipid droplet diameter as a function of lean and obese rats is plotted in Figure 3B. We found that, on average, the diameters of lipid droplets from obese Western rats were twofold larger than those from lean rat chow rats. This observation indicates that obesity increases the size of lipid droplets in mammary adipocytes.

Finally, we analyzed standard histologic tissue sections by CARS and SHG and compared the images with those obtained from fresh tissues. We found that most of the SHG collagen signal came from areas surrounding mammary ducts and terminal end buds in mammary gland tissue sections (Figure 4, A and B). Consistent with fresh tissue analysis, we observed that most collagen signal was found at the perimeter of the tumor mass (Figure 4, C and D). However, when comparing two-dimensional images of histology samples, we could not observe any correlation between obesity, the size of lipid droplets of adipocytes, and relative collagen content in mammary gland or tumor stroma. Given the fact that tissue sections were not sliced at the equatorial plane of the adipocytes, it became obvious that we could not compare the size of lipid droplets of adipocytes from one histologic tissue section with another. In addition, each tissue section was approximately 5 μm thick, less than the average size of a tumor cell ($\approx$10 μm); therefore, collagen fibrils present primarily outside the tumor mass could not be assayed. It is conceivable that serial sectioning of tissues and careful three-dimensional image reconstruction analysis of standard histology samples should allow evaluation of the impact of obesity on mammary gland and tumor stroma. However, given the tedious works involved with serial histology section analysis, a simpler alternative can be
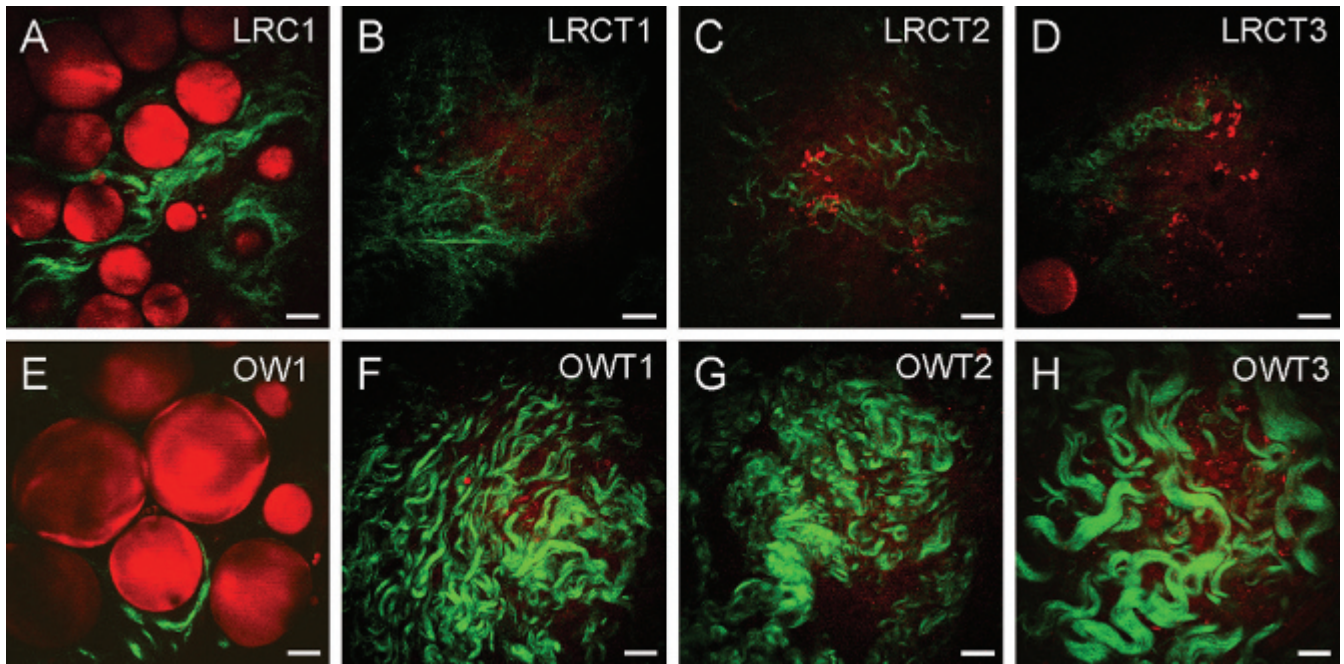
Figure 2. Coherent anti-Stokes Raman scattering imaging of adipocytes (*red*) and second harmonic generation imaging of collagen fibrils (*green*) to evaluate the impact of obesity on mammary gland and tumor stromal composition. Representative images (single frames) of (*A*) a mammary gland of one lean rat chow rat (LRC1), (*B–D*) mammary tumor stroma of three lean rat chow tumor rats (LRCT1, LRCT2, LRCT3), (*E*) a mammary gland of one obese Western rat (OW1), and (*F–H*) mammary tumor stroma of three obese Western tumor rats (OWT1, OWT2, OWT3). Images taken with a 60× water immersion objective. Scale bars = 25 μm.

found with NLO imaging, which has intrinsic three-dimensional sectioning capability.[11] Taken together, our results show that three-dimensional imaging of mammary tissues enabled analysis of the impact of obesity on mammary stroma not readily accessible by standard two-dimensional histologic evaluation.

Discussion

As breast tumors are mainly epithelial in nature, a majority of cancer studies have focused on activities occurring within epithelial cells.[5,6] However, increasing evidence indicates that surrounding stromal environment plays an integral role in mammary tumorigenesis.[6,8] Recent microarray gene expression analysis showed that secreted proteins from adipocytes induced epithelial cells to express genes crucial for proliferation, invasion, survival, and angiogenesis.[31] Supporting the importance of stromal composition in tumorigenesis, many reports have also demonstrated that overexpression of genes for ECM proteins was observed in aggressive mammary tumors.[32] Collagen type I, in addition to playing the role of structural support in the ECM, was found to be an important binding site for a large number of mitogenic and morphogenic growth factors.[33] Remodeling of collagen fibrils has been implicated in tumor-associated angiogenesis and invasiveness.[34] In light of such evidence,

the tissue organization theory proposes that tumorigenesis begins at the tissue level of biologic organization and is the result of altered stromal-epithelial interactions.[8] In this study, we combined NLO imaging techniques with an early-onset DIO animal model to evaluate how obesity alters mammary stromal composition, leading to the observed higher breast cancer incidence in obese individuals. We report direct correlations between obesity and average adipocyte size in mammary gland and between obesity and the ECM collagen content in mammary tumor stroma. Given the emerging importance of adipocytes and collagen for the growth and migration of mammary tumor cells, our observation provides the first visual evidence supporting the relationship between obesity and breast cancer risk.[35]

Our imaging data are in agreement with previous biochemical studies. A number of research groups have reported a significant decrease in collagen messenger ribonucleic acid transcripts (up to 90%) during adipogenesis, and lower collagen content led to a more permissive environment for the growth of adipocytes.[36,37] These studies lend support to our observation that collagen content decreases as adipocytes become larger in the mammary gland of obese rats. On the other hand, leptin, a protein hormone found to circulate at high concentration in obese animals, was reported to stimulate the expression
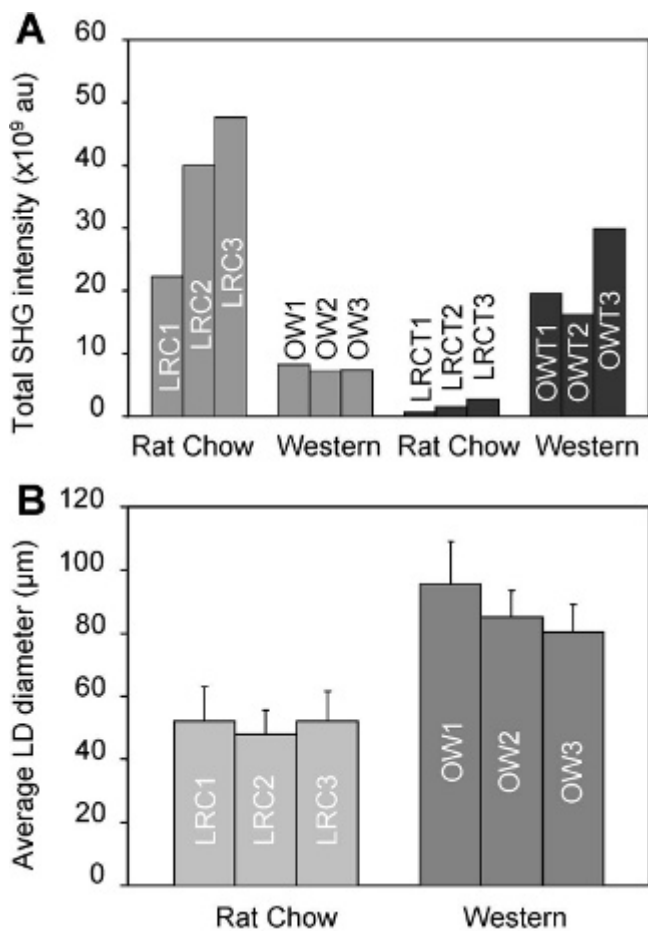
Figure 3. Analysis of the impact of obesity on mammary gland and tumor stromal composition. Mammary gland and tumor tissues of three rats from each animal group were analyzed for collagen content and diameter of lipid droplets (LD) of adipocytes. *A*, Total second harmonic generation (SHG) collagen intensity in mammary gland and tumor stroma. *B*, Average diameter of LDs of 100 adipocytes in mammary glands. Error bars represent the standard deviations from the average values.

of collagen type I in epithelial cells.[38–40] Additionally, transformation growth factor β_1, a surface receptor protein highly expressed in epithelial tumor cells, is an activator for collagen type I synthesis.[34,41] Such biochemical analyses indicate that whereas adipogenesis suppresses collagen synthesis in adipocytes, secreted adipocyte soluble factors stimulate collagen synthesis in epithelial cells. A high level of circulating leptin in obese rats supports our observation of higher collagen content in the mammary tumor stroma of obese rats compared with lean rats.[35,38–40] Low collagen content in the mammary gland and high collagen content in the mammary tumor stroma of obese rats could be due to the relative abundance of adipocytes and epithelial cells, the main sources of collagen in these tissues, respectively. Although it remains unclear how obesity induces mammary tumorigenesis, our data clearly show that obesity
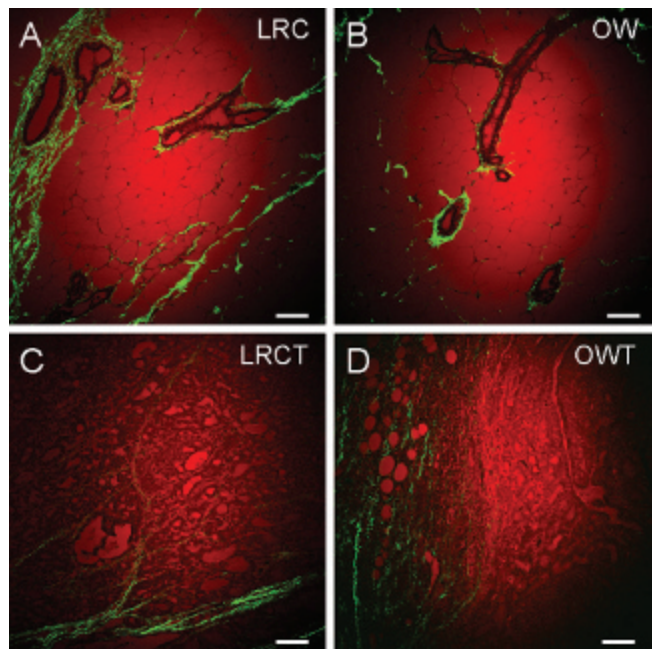

Figure 4. Coherent anti-Stokes Raman scattering imaging of lipid (*red*) and second harmonic generation imaging of collagen fibrils (*green*) of standard histologic tissue sections. Histology of the mammary gland of (*A*) a lean rat chow rat (LRC), (*B*) an obese Western rat (OW), and the mammary tumors of (*C*) a lean chow tumor rat (LRCT) and (*D*) an obese Western tumor rat (OWT). Images taken with a 20× air objective. Scale bars = 75 μm.

promotes a stromal environment conducive for the proliferation and migration of mammary epithelial cells.

In summary, by combining SHG, TPEF, and CARS into a single multimodal platform, we have shown the unique advantages of NLO imaging of mammary cancer in fresh tissues. Especially, CARS and SHG allowed us to visualize tumor cell and the significant components of tumor stroma, such as blood capillaries, adipocytes, and collagen fibrils, without the need for labeling. Given the capability of multiphoton microscopy for live animal imaging,[12,13,15,21] it is conceivable that mammary tumor development can be imaged as a function of time noninvasively. Future work that correlates mammary tumor stromal composition and structural organization with histologic evaluation of tumor aggressiveness should lay the foundation for the use of multimodal NLO imaging to diagnose mammary tumor phenotype.

Acknowledgments

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Supplementary information for "Nonlinear Optical Imaging to Evaluate the Impact of Obesity on Mammary Gland and Tumor Stroma", Le *et al.*
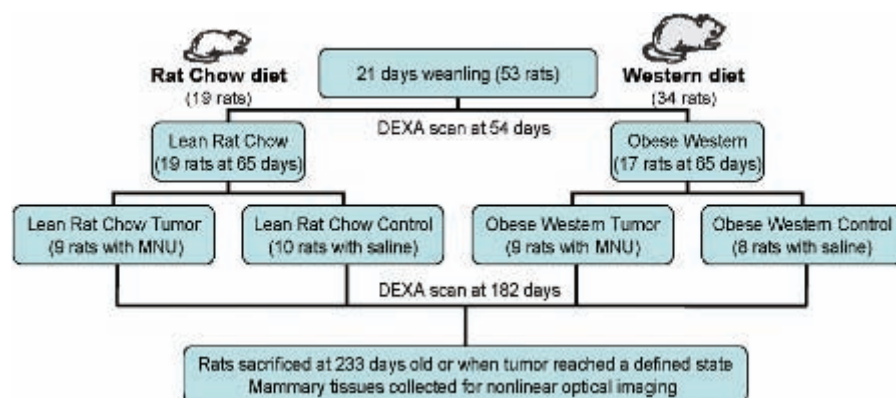

Figure S1. An early-onset diet-induced obesity breast cancer animal model. Of the 34 rats placed on the Western diet, 17 rats became obese. DEXA = dual-energy x-ray absorptiometry; MNU = methylnitrosourea.
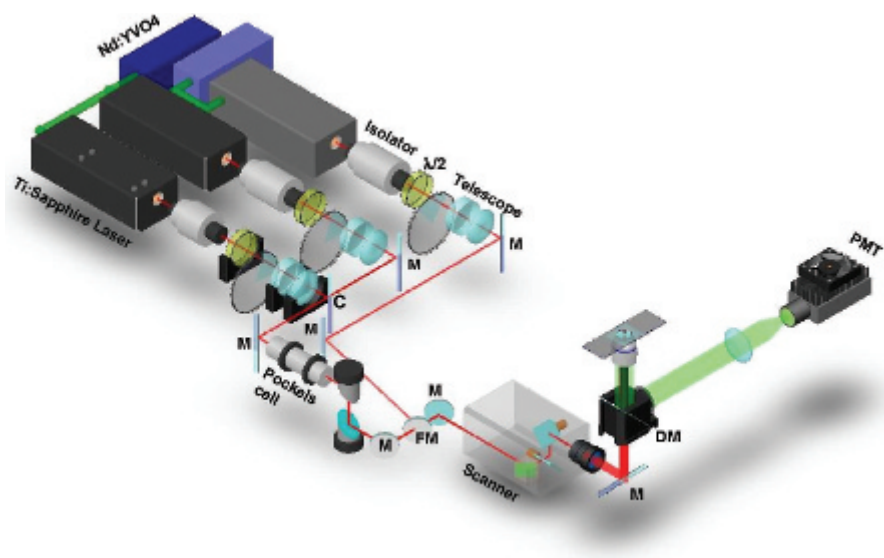

Figure S2. A multimodal multiphoton microscope that allows coherent anti-Stokes Raman scattering, second harmonic generation, and two-photon excitation fluorescence imaging on the same platform. C = combiner; DM = dichroic mirror; FM = flipper mirror; M = mirror; PMT = photomultiplier tube.
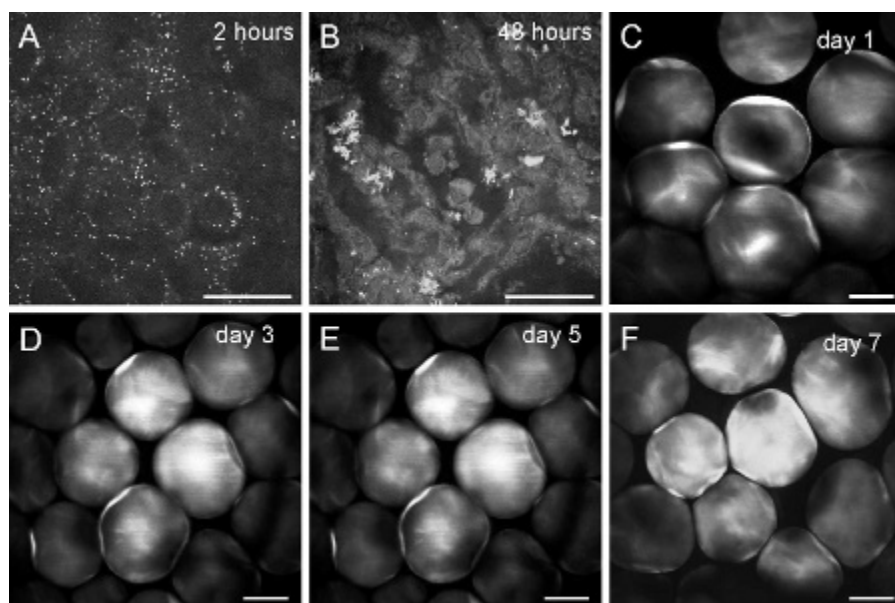
Figure S3. Stability of tumor cells of mammary tumor tissues and of adipocytes of mammary gland tissues in maintenance media. Tumor cells of mammary tumor tissues imaged with coherent anti-Stokes Raman scattering (CARS) at (*A*) 2 hours and (*B*) 48 hours after tissue collection. Adipocytes of mammary gland tissues imaged with CARS at (*C*) 1 day, (*D*) 3 days, (*E*) 5 days, and (*F*) 7 days after tissue collection. Scale bars = 25 μm.

Stability of Mammary Tumor and Mammary Gland Tissues

To analyze the stability of tumor cells of mammary tumor tissues and adipocytes of mammary gland tissues in maintenance media, we performed CARS imaging of tumor cells and adipocytes over time. During the first few hours after tissue collection, tumor cells were filled with lipid droplets, packed closely together, and had an average size of approximately 10 μm (Figure S3A). However, the lipid droplets in tumor cells disappeared rapidly over the next few hours. In addition, tumor cells shrunk to an average size of approximately 5 μm and gaps formed between cells at 48 hours after tissue collection (Figure S3B). In contrast, adipocytes remained stable for over 1 week in maintenance media (Figure S3, C–F). Learning from these observations, we imaged mammary tumor tissues immediately after collection or kept the tissues in liquid nitrogen for imaging at a later time. We found that

tumor tissues stored in liquid nitrogen maintained the characteristics of freshly collected tissues (data not shown). We imaged mammary gland tissues immediately or up to 7 days after tissue collection. Mammary gland tissues were kept in maintenance media in an incubator set at 37°C and 5% CO_2. We found that freezing mammary gland tissues caused distortion to the shape of adipocytes (data not shown).

Labeling of Mammary Tumor Cells with Sp-DiOC18

Mammary tumor tissues were incubated in maintenance media mixed with Sp-DiOC18 (3 μg/mL final concentration; Molecular Probes, Eugene, OR) for 2 hours in a 37°C incubator with 5% CO_2. Tissues were washed three times and then incubated in maintenance media for 1 hour to remove excess DiOC18 before imaging with CARS and TPEF.
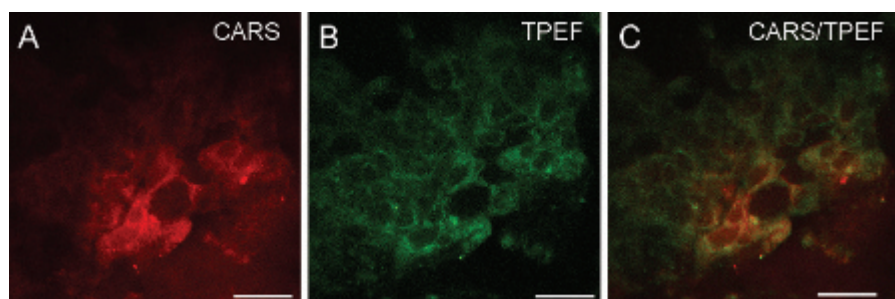

Figure S4. Mammary tumor cells labeled with DIOC18 and imaged with (*A*) coherent anti-Stokes Raman scattering (CARS) (*red*) and (*B*) two-photon excitation fluorescence (TPEF) (*green*). *C*, Overlay of CARS and TPEF images. Images taken with a 60× water immersion objective. Scale bars = 25 μm.
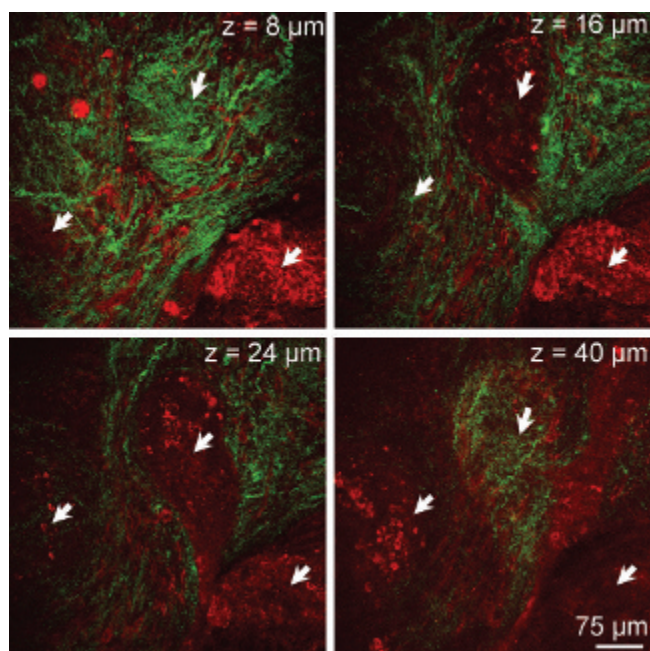
Figure S5. Imaging the orientation of collagen fibrils (*green*) with second harmonic generation and tumor mass with coherent anti-Stokes Raman scattering microscopy. *Arrows* point to tumor areas. Collagen fibrils appear to define the boundaries of the tumor mass. Images taken at the same area along the axial axis (z) using a 20× air objective.

Table S1. Evaluating the Impact of Obesity on Mammary Gland and Tumor Stromal Composition

Animal Groups (3 rats/group)	Total Collagen Content (SHG intensity, $\times 10^9$ arbitrary units)	Diameter of Adipocytes' LD (CARS imaging, μm)
Lean rat chow		
LRC1	22.3	52.1 ± 10.9
LRC2	39.9	47.9 ± 7.8
LRC3	47.0	52.2 ± 9.4
Obese Western		
OW1	8.2	95.4 ± 13.7
OW2	7.2	85.1 ± 8.7
OW3	7.3	80.3 ± 8.9
Lean rat chow tumor		
LRCT1	0.6	NA
LRCT2	1.5	NA
LRCT3	2.7	NA
Obese Western tumor		
OWT1	19.5	NA
OWT2	16.2	NA
OWT3	29.9	NA

CARS = coherent anti-Stokes Raman scattering; LD = lipid droplet; NA = not available; SHG = second harmonic generation.
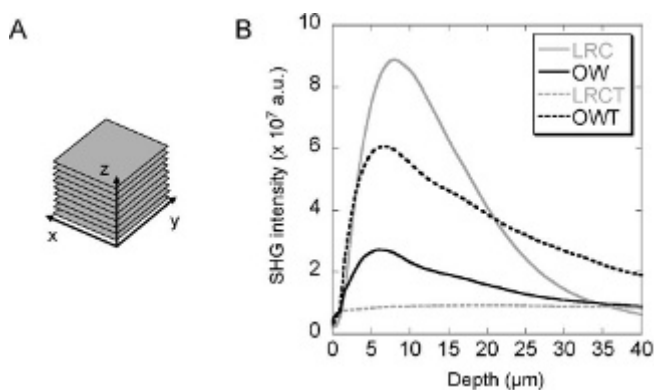

Figure S6. Analysis of mammary gland and tumor stromal composition of collagen fibrils type I. *A*, An analysis volume is defined with the dimensions of 250 μm (x) × 250 μm (y) × 40 μm (z), with z = 0 being the coverslip and tissue interface. For each analysis volume, 81 frames along the z-axis with a fixed step size of 0.5 μm were acquired. Nine different volumes were evaluated, and the total second harmonic generation (SHG) intensity from 729 frames was used to infer the mammary stromal collagen content of each animal. *B*, SHG intensity as a function of depth of one representative analysis volume in each animal. LRC = lean rat chow; LRCT = lean rat chow tumor; OW = obese Western; OWT = obese Western tumor.
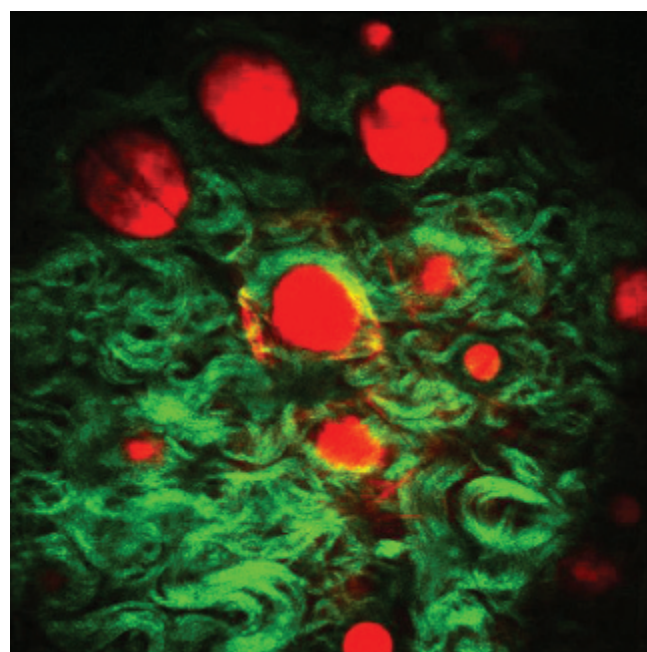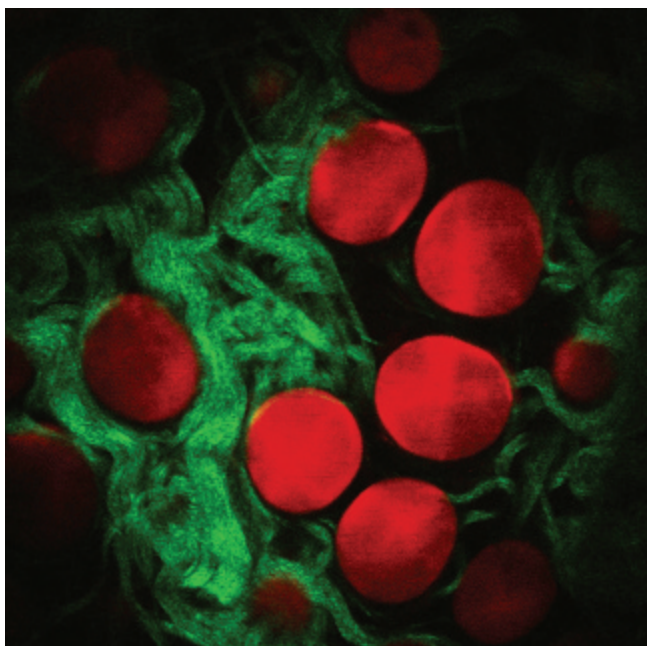

Supplementary Video S1. Three-dimensional imaging of adipocytes and blood capillaries with coherent anti-Stokes Raman scattering (*red*) and collagen fibrils type I with second harmonic generation (*green*) microscopy. The video is a collage of 30 frames taken at a 0.5 μm step size along the axial axis (z). A single frame at z = 6 μm is presented as Figure 1A.
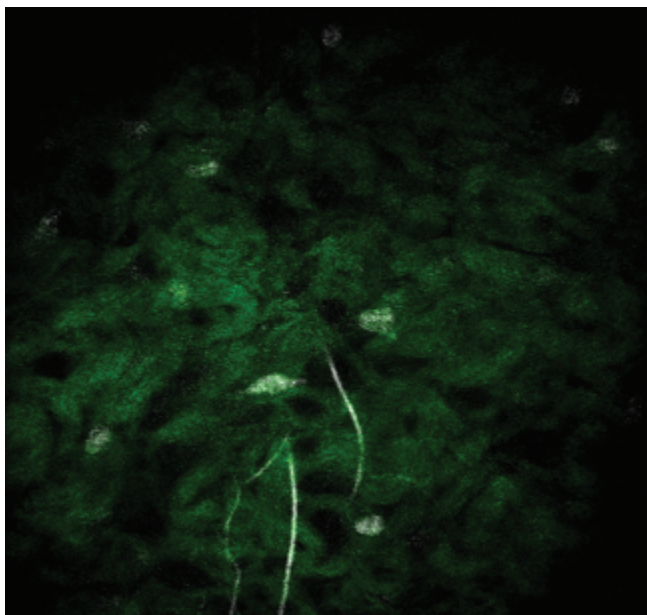
Supplementary Video S2. Three-dimensional imaging of collagen fibrils with second harmonic generation (*green*) and fluorescein isothiocyanate conjugated isolectin B$_4$–labeled blood capillaries and activated macrophages with two-photon excitation fluorescence (*gray*) microscopy. The video is a collage of 150 frames taken at a 0.2 μm step size along the axial axis (z). A single frame at z = 6 μm is presented as Figure 1B.
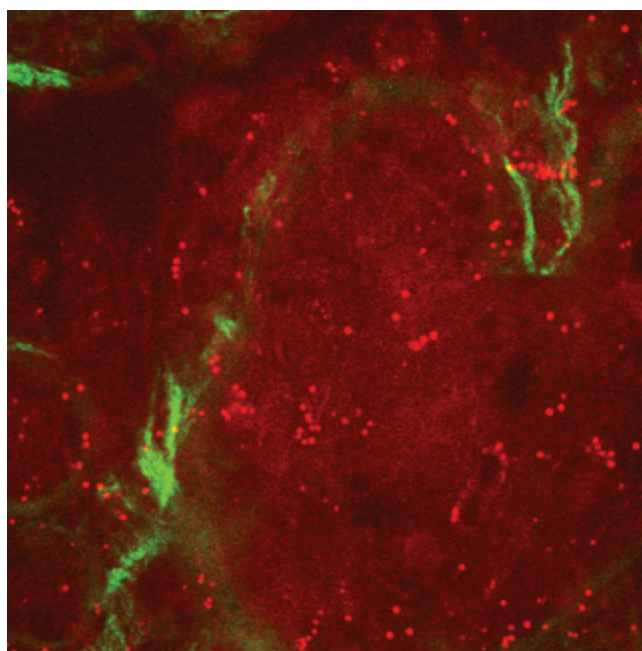
Supplementary Video S4. Three-dimensional imaging of mammary tumor cells with two-photon excitation fluorescence (*red*) and collagen fibrils type I (*green*) with second harmonic generation microscopy. The video is a collage of 70 frames taken at a 0.5 μm step size along the axial axis (z). Three single frames are presented as Figure 1, D to F.

Supplementary Video S3. Three-dimensional imaging of adipocytes with two-photon excitation fluorescence (*red*) and collagen fibrils type I with second harmonic generation (*green*) microscopy. The video is a collage of 30 frames taken at a 0.5 μm step size along the axial axis (z). A single frame at z = 8 μm is presented as Figure 1C.